2, but retained expression of IL-2R, thus recapitulating the classical anergy pathway. In contrast, direct treatment with RTLs did not induce proliferation, Th1 cytokine responses, or IL-2R expression, but did strongly induce IL-10 secretion (Fig. 24). RTL pretreatment partially reduced proliferation responses and Th1 cytokine secretion, but did not inhibit IL-2R expression upon restimulation of the T cells with APC/antigen. Importantly, these T cells continued to secrete IL-10. Thus, it is apparent that the focused activation of T cells through antibody crosslinking of the CD3-chain had vastly different consequences than activation by RTLs presumably through the exposed TCR surface. It is probable that interaction of the TCR with MHC/antigen involves more elements and a more complex set of signals than activation by crosslinking CD3-chains, and the results described herein indicate that signal transduction induced by anti-CD3 antibody may not accurately portray ligand-induced activation through the TCR. Thus, CD3 activation alone likely does not comprise a normal physiological pathway.

REMARKS

These amendments are made solely to remove the references to Fig. 25. Page 82 is further amended to incorporate the description for Fig. 25, previously found on page 14 of the specification. No new matter is added.

CONCLUSION

Examination of the subject application is respectfully requested. If any minor matters remain to be addressed prior to examination, the examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

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Marked-Up Version of Amended Specification

Pursuant to 37 C.F.R. §§ 1.121(b)-(c)

Please see attached pages.

represent mean ± SEM. Clone MR#3-1 showed initial proliferation to anti-CD3, but not to RTLs.

~~Fig. 25 is a set of graphs showing IL-10 cytokine production induced by RTL pre-treatment was maintained after stimulation with APC/peptide. T cells showed a reduced ability to proliferate and produce cytokines after anti-CD3 or RTL treatment, and the RTL effect was antigen and MHC specific. IL-10 was induced only by specific RTLs, and Il-10 production was maintained even after restimulation with APC/antigen. T cell clones were cultured at 50,000 cells/well with medium, anti-CD3, or 20 μM RTLs in triplicate for 48 hours, and washed once with RPMI. After the wash, irradiated (2500 rad) frozen autologous PBMC (150,000/well) plus peptide-Ag (MBP-85-99 at 10 μg/ml) were added and the cells incubated for 72 hr with ^{3}H-thymidine added for the last 18 hr. Each experiment shown is representative of at least two independent experiments. Bars represent mean ± SEM. For cytokine assays, clones were cultured with 10 μg/ml anti-CD3 or 20 μM RTL303 or RTL311 for 48 hours, followed by washing with RPMI and re-stimulation with irradiated autologous PBMC (2500 rad, T:APC=1:4) plus peptide-Ag (10 ∝g/ml) for 72 hours. Cytokines (pg/ml) profiles were monitored by immunoassay (ELISA) of supernatants. Each experiment shown is representative of at least three independent experiments. Bars represent mean ± SEM.~~

Sequence Listing

The sequence listing appended hereto includes sequences as follows:

SEQ ID NO:1: the nucleic acid of a single chain ß1α1 expression cassette.

SEQ ID NO:2: the amino acid sequence encoded by the construct shown in SEQ ID NO:1.

SEQ ID NO:3: the nucleic acid sequence of an antigen/linker insert suitable for insertion into the expression cassette shown in SEQ ID NO:1.

SEQ ID NO:4: the amino acid sequence encoded by the sequence shown in SEQ ID NO:3.

markers. RTL treatment induced only subtle increases in apoptotic changes as quantified using Annexin V staining and these were not Ag-specific. Treatment of T cell clones with RTLs did not induce proliferation when added in solution, immobilized onto plastic microtiter plates, nor in combination with the addition of anti-CD28.

Upon activation with APC plus Ag, clone MR#3-1 (MBP85-99 specific) and MR#2-87 (CABL specific) showed classic Th1 cytokine profiles that included IL-2 production, high IFN-γ and little or no detectable IL-4 or IL-10. As is shown in Fig 24A, activation through the CD3-chain with anti-CD3 antibody induced an initial burst of strong proliferation and production of IL-2, IFN-γ, and surprisingly, IL-4, but no IL-10. In contrast, upon treatment with RTL303, clone MR#3-1 continued production of IFN-γ, but in addition dramatically increased its production of IL-10 (Fig. 24A). IL-10 appeared within 24 hours after addition of RTL303 and its production continued for more than 72 hours, to three orders of magnitude above the untreated or RTL311 treated control. In contrast, IL-2 and IL-4 levels did not show RTL induced changes (Fig. 24A). Similarly, after treatment with RTL311, Clone MR#2-87 (CABL specific) also showed a dramatic increase in production of IL-10 within 24 hours that continued for greater than 72 hours above the untreated or RTL303 treated control (Fig. 24B). Again, IL-2 and IL-4 levels did not show detectable RTL induced changes, and IFN-γ production remained relatively constant (Fig. 6B). The switch to IL-10 production was exquisitely Ag-specific, with the clones responding only to the cognate RTL carrying peptide antigen for which the clones were specific. The DR7 homozygous T cell clone CP#1-15 specific for MBP-85-99 showed no response to DR2-derived RTLs, indicating that RTL induction of IL-10 was also MHC restricted.

To assess the effects of RTL pre-treatment on subsequent response to antigen, T cell clones pretreated with anti-CD3 or RTLs were restimulated with APC/peptide, and cell surface markers, proliferation and cytokine production were monitored. T cell clones were cultured at 50,000 cells/well with medium, anti-CD3, or 20 μM RTLs in triplicate for 48 hours, and washed once with RPMI. After the wash, irradiated (2500 rad) frozen autologous PBMC (150,000/well) plus peptide-Ag (MBP-85-99 at 10 μg/ml) were added and the cells incubated for 72 hr with ^{3}H-thymidine added for the last 18 hr. For cytokine assays, clones were cultured with 10 μg/ml anti-

CD3 or 20 μM RTL303 or RTL311 for 48 hours, followed by washing with RPMI and re-stimulation with irradiated autologous PBMC (2500 rad, T:APC=1:4) plus peptide-Ag (10 μg/ml) for 72 hours. Cytokines (pg/ml) profiles were monitored by immunoassay (ELISA) of supernatants. RTL pre-treatment had no effect on the cell surface expression levels of CD25, CD69 or CD134 (OX40) induced by restimulation with APC/peptide compared to T cells stimulated with APC/peptide that had

never seen RTLs, and there were no apoptotic changes observed over a 72 hour period using Annexin V staining (data not shown).

As anticipated, anti-CD3 pretreated T cells were strongly inhibited, exhibiting a 71% decrease in proliferation and >95% inhibition of cytokine production, with continued IL-2R (CD25) expression (Table 6~~; Fig. 25~~), a pattern consistent with classical anergy (Elder et al., 1994). T cells showed a reduced ability to proliferate and produce cytokines after anti-CD3 or RTL treatment, and the RTL effect was antigen and MHC specific. IL-10 was induced only by specific RTLs, and IL-10 production was maintained even after restimulation with APC/antigen.

Table 6. Ag-specific inhibition of T cell clones by pre-culturing with RTLs.

Donor 1					
Clone #3-1		**Pre-Cultured with RTL303***		**Pre-Cultured with RTL311**	
	Untreated	20 μM	10 μM	20 μM	10 μM
+APC**	439 ± 221	549 ± 70	406 ± 72	491 ± 50	531 ± 124
+APC+MBP-85-99 (10 μg/ml)	31725 ± 592	18608 ± 127	29945 ± 98	35172 ± 41	32378 ± 505
Inhibition (%)	-	**-42.3 (p<0.01)**	**-5.6**	**0**	**0**
Clone #2-87					
+APC	1166± 24	554 ± 188	1229 ± 210	1464 ± 281	1556 ± 196
+APC+C-ABL-b2a3 (10 μg/ml)	11269 ± 146	11005 ± 204	14298 ± 1669	5800 ± 174	7927 ± 575
Inhibition (%)	-	**0**	**0**	**-57.0 (p<0.001)**	**-36.9 (p<0.01)**
Donor 2					
Clone #1-15					
+APC	258 ±± 48	124 ±7	ND	328 ± 56	ND
+APC+MBP-85-99 (10 μg/ml)	7840 ± 1258	7299 ± 1074	ND	8095 ± 875	ND
Inhibition (%)	-	**- 5.1**		**0**	

*Soluble RTL303 or RTL311 were co-cultured with T cell clones at 200,000 T cells/200 μl medium for 48 hours followed by washing twice with RPMI 1640 prior to the assay. **2 x 10^5 irradiated (2500 rad) autologous PBMC were added at ratio 4:1 (APC:T) for 3 days with ^{3}H-Thymidine incorporation for the last 18 hr. The p values were based on comparison to "untreated" control.

Clone MR#3-1 showed a 42% inhibition of proliferation when pretreated with 20 μM RTL303, and clone MR#2-87 showed a 57% inhibition of proliferation when pretreated with

20 μM RTL311 (Table 6~~; Fig. 25~~). Inhibition of proliferation was also MHC class II-specific, as clone CP#1-15 (HLA-DR7 homozygous donor; MBP85-99 specific) showed little change in proliferation after pre-treatment with RTL303 or RTL311 (Table I). Clone MR#3-1 pretreated with RTL303 followed by restimulation with APC/Ag showed a 25% reduction in IL-2, a 23% reduction in IFN-γ and no significant changes in IL-4 production~~ (Fig. 25)~~. Similarly, clone MR#2-87 showed a 33% reduction in IL-2, a 62% reduction in IFN-γ production, and no significant change in IL-4 production. Of critical importance, however, both RTL-pretreated T cell clones continued to produce IL-10 upon restimulation with APC/peptide~~ (Fig. 25)~~.

The results presented above demonstrate clearly that the rudimentary TCR ligand embodied in the RTLs delivered signals to Th1 cells and support the hypothesis of specific engagement of RTLs with the αβ-TCR signaling. Signals delivered by RTLs have very different physiological consequences than those that occur following anti-CD3 antibody treatment.

In the system described herein, anti-CD3 induced strong initial proliferation and secretion of IL-2, IFN-γ, and IL-4 (Fig. 24). Anti-CD3 pre-treated T cells that were restimulated with APC/antigen had markedly reduced levels of proliferation and cytokine secretion, including IL-2, but retained expression of IL-2R, thus recapitulating the classical anergy pathway~~ (Fig. 25)~~. In contrast, direct treatment with RTLs did not induce proliferation, Th1 cytokine responses, or IL-2R expression, but did strongly induce IL-10 secretion (Fig. 24). RTL pretreatment partially reduced proliferation responses and Th1 cytokine secretion, but did not inhibit IL-2R expression upon restimulation of the T cells with APC/antigen. Importantly, these T cells continued to secrete IL-10~~ (Fig. 25)~~. Thus, it is apparent that the focused activation of T cells through antibody crosslinking of the CD3-